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We propose to generate a novel inducible bitransgenic system to examine the effect of the expression of a target oncogene, the polyoma middle T antigen, on the onset of mammary epithelium hyperplasia. Using this inducible system, the specific expression of a transgene can be initiated by the administration of an external compound. To establish such a regulatable system, we have introduced the regulator and the inducible target separately into mouse embryos to generate transgenic mouse lines. To target the expression of the regulator in mammary glands, we have placed the regulator under the control of the MMTV-LTR. Transgenic lines of the regulator and the target, polyoma middle T antigen have been generated. These lines are currently being crossed to generate bitransgenic lines and the expression of the target oncogene will be induced by administration of progesterone antagonists. The regulated expression of polyoma middle T antigen in the mammary gland of bitransgenic mice will allow the investigation of the potential protective effects of ovarian steroid hormones on the development of mammary tumors in response to specific oncogene expression. The success of this approach may have far-reaching effects on the future understanding of the mechanisms of oncogenesis in mammary epithelium.

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TABLE OF CONTENTS

INTRODUCTION

Significance

Breast cancer continues to be a prevailing disease among women in the United States. Though numerous models for breast cancer have been developed, the etiology of the disease remains obscure. The transgenic mice technology has offered a means of targeting oncgoenes to the mammary glands to help better understand the role of these oncogenes play in the development of breast cancer. However, the constitutive expression of oncogene does not allow the assessment of the critical time points in which the mammary glands are most susceptible to oncogenesis. Thus, it is most imperative that a regulatable system be established to turn genes on and off, rendering it feasible to define the role of a candidate oncogene plays in mammary gland oncogensis.

A novel inducible bitransgenic mice system is being generated to meet this goal. It is made up of two lines of mice. The first line of mice, the regulator mice, carries a chimeric transcription factor which is activated by an exogenous ligand is targetted to mammary gland via the MMTV promoter. This chimeric regulator consists of three functional domains, an activation domain from HSV-VP16, a Gal4 DNA binding domain and a modified version of the progesterone receptor ligand binding domain which is responsive to anti-progestins (ie RU486) but not to progestins. The second line of mice, the target mice, carries an oncogene under the control of four yeast transcription factor GAL4 binding sites (refereed as 17X4) where the regulator can bind to. Two mimimal promoters have been chosen for the target genes namely, the E1B TATA and theTK promoter. When these two transgenic lines are crossed to create the bitransgenic mice , the regulator can then bind to the 17mer Gal4 recognition sequences upstream of the target oncogene and induce the expression of the target oncogene with the administration of RU486. The expression of an oncogene in the bitransgeneic mice can be induced by RU486 at a specific window during development and the effects of this oncogene on mammary gland oncogenesis can be monitored. With this system one can also study the interaction between hormones and the target oncogene during mammary gland development.

To test our regulatable system, we will be using the polyma mddle T antigen (pMT) as our target oncogene in our breast cancer model. pMT is a potent oncogene which has been to cause multifocal tumours in the mammary glands of female mice. This target will be regulated by our transactivator, which will be targeted for expression in the mammary galnds.

BODY

Construction of MMTVGLVP1 Transgenic Vector

To facilitate mammary gland-specific expression, the regulator is put under the control of the MMTV promoter. The regulator is comprised of a chimeric construction of HSV-VP16 activation domain, Gal4 DNA binding domain and the modified progesterone receptor ligand binding domain. A BamHI-SalI fragment of the KCR fragment made up of partial exon II, intron II, exon III with 3' untranslated region of the rabbit betaglobin gene, sequences spanning the SV40 polyA and parts of the pBR322 plasmid sequences has been cloned into corresponding sites in the bluescript vector KSII from Stratagene. The use of the KCR fragment is to facilitate proper splicing and expression of the regulator transgene. A blunt-ended fragment of the regulator was then cloned into the gap-filled EcoRI site in Exon III of the KCR construct. A 2.3 Kb BamHI fragment of the MMTV-LTR was subsequently cloned into the BamHI site of the KCR-regulator construct to give the resulting MMTV-KCR-GLVP1 transgenic vector as diagrammed in Figure 1. The orientation of the construct has been verified by restriction enzyme analysis.

Transfection to test functionality of the MMTV-KCR-GLVP1 transgenic vector

To test for the functionality of the MMTV-KCR-GLVP1 transgenic construct, transient transfection assays in monkey kidney CV1 cells were used. This transgenic construct was transfected with various combinations of the progesterone receptor and the 17X4 TK-chloramphenicol acetyltransferase(CAT) reporter in the presence and absence of RU486, a progesterone antagonist. As a control, SV2CAT reporter was added to assess transfection efficiency.

As shown in Figure 2, when the MMTV-KCR-GLVP1 transgenic construct was cotransfected with the progesterone receptor to activate the MMTV promoter and the reporters, CAT activity is high only in the presence of RU486, but not in its absence. It is worth noting that the basal activity is seen with the 17X4 TK-CAT reporter itself. To lower the basal activity, we use the 17X4 E1bTATA reporter which yields a much lower basal activity. However, the maximal levels of induction is also lower as compared to the reporter with the TK promoter (data not shown). From this assay, it is clear that the MMTV-KCR-GLVP1 regulator is capable of inducing expression of the 17X4 CAT reporters in the presence of the ligand, RU486..

Generation of MMTV-KCR-GLVP1 regulator transgenic mice

To facilitate high recombination and integration frequencies in the germline DNA of fertilized one-cell embryos, the MMTV-KCR-GLVP1 construct was linearized with the NotI and Acc65I restriction enzymes. The purified linearized fragment was then micro injected into fertilized one-cell embryos derived from super ovulated female mice donors and then the injected embryos were then transferred into female recipients that have been mated with vasectomized males. When the pups have reached 3 weeks

old, tail cuts were made and the genomic DNA derived were then screened for the presence of the MMTV-KCR-GLVP1 transgene by Southern analysis with a probe corresponding to the whole regulator (Fig.3).

Six founders were obtained and they were breeded to generate respective litters.

Assay for the expression of the regulator transgene

Northern analyses were used to assay for the expression of the regulator transgene. Mammary gland biopsies were performed on 10-day lactating females that have just given birth to pups and total RNA were isolated from these tissues. A probe spanning the entirety of the regulator was used to hybridize with RNA immobilized on the membranes. In vitro-transcribed regulator RNA were spiked with total RNA from lactating females not bearing the MMTV-KCR-GLVP1 transgene to serve as positive controls. None of the 5 lines express the regulator through repeated northern analyses. Therefore, either the regulator is not expressed or it is expressed at low level below the sensitivity of the Northern analysis.

To examine whether the regulator is expressed at low levels, RT-PCR was employed. Two primers spanning intron II of the KCR construct were used to differentiate the amplification of contaminating genomic DNA versus cDNA generated via reverse transcription from total RNA as schematically depicted in figure 4. A correctly expressed transcript should yield a 230bp amplified product from reverse transcription, while the contaminating DNA will generate a product of about 850bp. Total RNA from monkey kidney SV40-transformed COSM6 cell transfected with both the progesterone receptor and the MMTV-KCR-GLVP1 construct and supplemented with R5020 were used as positive controls for the regulator expression. Figure 5 shows that only the 7134 Fo line showed weak expression of the expected size of 230 bp product from lactating mammary gland RNA out of the 3 lines tested. This line has been bred to generate more progeny. One line is currently being tested by RT-PCR for expression while the remaining two lines are still breeding to generate females that they could be assayed for expression when they become pregnant. The current status of the MMTV-KCR-GLVP1 mice is summarized in table 1.

Generation of the polyoma middle T target transgenic mice

The unique feature of this bitransgenic model is the fact that the expression of the target is modulated by the regulator. The polyoma middle T(pMT) antigen has been chosen as the target transgene due its high potency of generating tumors with few additional genetic mutations.

The blunt-ended HindIII-EcoRI pMT minigene was cloned into the EcoRV site of both the 17X4 TK and E1bTATA target vectors. The validity of these constructs were confirmed by both restriction enzyme analyses and sequencing of the ligation-junctions. A schematic representation of these vectors are seen in Figure 6.

Functionality of the pMT targets

The ability of the targets to be induced is an important requisite for the success of this bitransgenic system. To test the inducibility of the targets, in vitro foci assays were performed using cloned-rat embryo fibroblasts(CREF) cells. The MMTV-KCR-GLVP1 construct was cotransfected with various combinations with the progesterone receptor and the 17X4 TK-pMT target into these CREF cells. The cells were split 2 days later and the media changed twice a week. RU486 and R5020 were added according to certain sets of the cells. Preliminary results showed that the 17X4 TK-pMT target alone generated few foci whereas when MMTV-KCR-GLVP1 was cotransfected with both the progesterone receptor and the 17X4 TK pMT vector in the presence of both RU486 and R5020, numerous foci were seen. This quick assay demonstrates that the 17X4 TK-pMT target is capable of being induced by the regulator.

The 17X4 E1bTATA-pMT target is currently being tested for inducibility.

Generation of 17X4 pMT target mice

Linearized fragments from both the 17X4-TK-pMT and E1b-TATA-pMT target vectors were generated with HindIII and Asp718 restriction enzyme digestion and the purified fragments were then micro-injected into fertilized one-celled fvb embryos.

At the present moment, southern screening of 17X4 TK-pMT target mice showed two founders. These founders were being bred to expand the line and subsequently crossed with the MMTV-KCR-GLVP1 7134 line to generate potential bitransgenic mice.

ONGOING EXPERIMENTS

Generate more MMTV-KCR-GLVP transgenic mice

It is imperative to obtain a regulator line that expresses the transgene at least at a moderate level detectable by northerns. To this aim, more lines of the MMTV-KCR-GLVP1 lines will have to be generated.

Multiple factors might contribute to the inability of our regulator construct to be expressed in transgenic mice. It is posible that the presence of 275 bp of pBR322 sequence at the end of our construct inhibits its expression. To get around that problem, modifications to the KCR vector has been made to get rid of the unwanted plasmid sequences and replaced with the bovine growth hormone(bGH) polyA sequences. This modified MMTV-KCR-HAGLVP1bGHpA vector also has an hemagglutin(HA)-tagged epitope engineered into the regulator. The purpose of this is to aid in the detection of the regulator protein. This construct has been injected and tail-cuts of potential Fo offspring are currently being carried out. Figure 7 depicts this modified regulator construct.

A MMTV-KCR construct from Dr. Brigid Hogan has also been acquired for our purposes here. This construct uses the native rabbit betaglobin(rbg) polyA sequences and the fact that the initial KCR used has two polyA sequences, one from rbg and the other from the SV40 small t intron polyA. The HA-tagged regulator has been cloned into this MMTV-KCR construct as diagrammed in Figure 8 and is presently been microinjected. It is hoped that with these modifications, a high-expressing-regulator can be obtained.

Modifications to the regulator

The desirability of a good regulator lies in the fact that it has a low basal activity in the absence of its activator-ligand and that it provides high induction in the presence of the ligand. Improvements to the regulator has been made by moving the VP16 domain to the C-terminal part of the protein and extending amino acid residues to the ligand binding domain of the progesterone receptor. Preliminary tranfection data showed that this modification renders the regulator to show a strong induction in the presence of RU486 with respect to CAT reporter activity and to show low CAT reporter activity in the absence of RU486.

Efforts to generate the MMTV-KCR construct with this improved regulator will be carried out . Once available it will be microinjected and potential Fo offsprings will be generated. Figure 9 denotes the schematic representation of this regulator.

Generation of bitransgenic MMTV-KCR-GLVP and 17X4 Int-2 mice

The 17X4 Int-2 mice generated by Phil Leder's lab has been acquired and is currently being quarantined. Efforts will be made to expand this line to generate more that will be crossed with the MMTV-KCR-GLVP regulator line to generate bigenics. This will also be a good system to use because Int-2 has been demonstrated to generate tumors in mammary glands in Phil Leder's bitransgenic mice and will provide a good model system to test our regulatable system.

CONCLUSIONS

We have generated a regulator transgenic mice line which is expressed at low levels in the mammary gland. This line is currently mated to the target polyoma middle T target transgenic mice. Once the bi-transgenic line is generated, the inducible expression of polyoma middle T antigen will be assessed with and without administration of RU486. More transgenic lines capable of expressing higher levels of regulator are being generated. The production of a good regulator is vital to the success of this bitransgenic system and efforts aimed at achieving this goal is underway.

REFERENCES

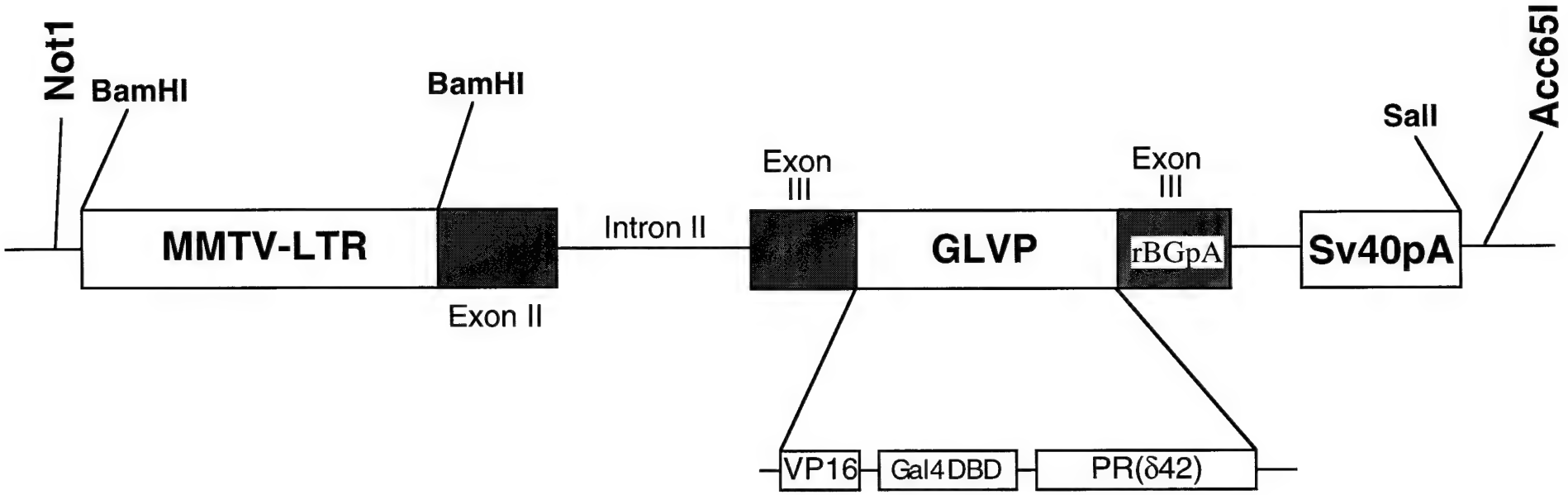
None

APPENDIX

Figures 1 - 9.

Table 1

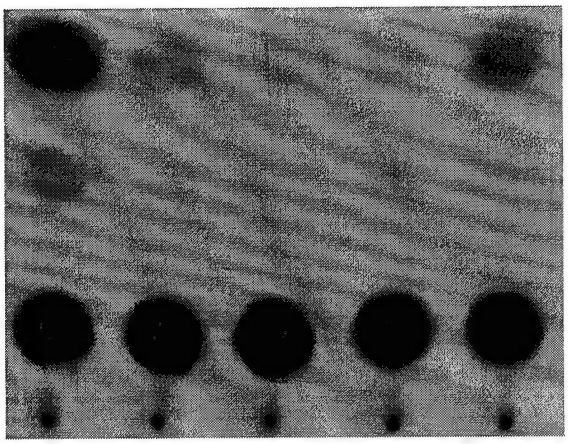
MMTV-KCR-GLVP1 transgenic vector



Gal4DBD: Gal4 DNA binding domain
rBGpA: rabbit betaglobin poly A
SV40pA: SV40 polyA

Figure 1

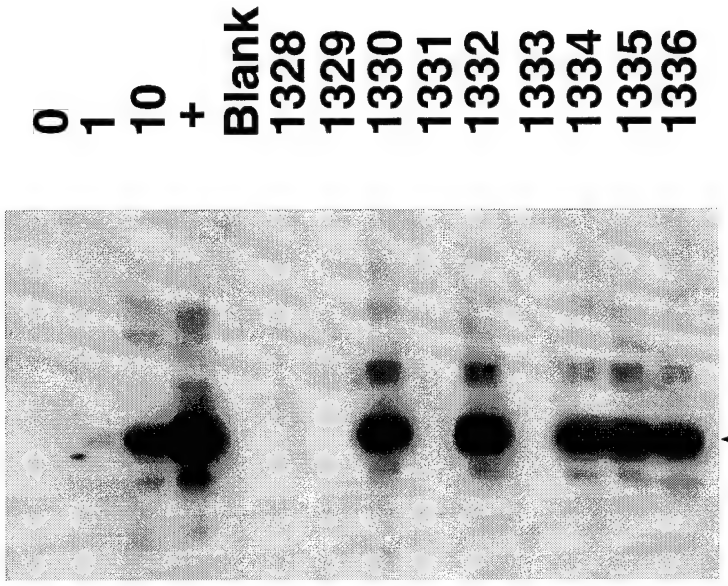
Induction of CAT reporter expression by MMTV-KCR-GLVP1



SV2CAT	+	-	-	-	-
RSV-hPR	-	-	-	+	+
17X4 TKCAT	-	+	+	+	+
MMTV-KCR-GLVP1	-	-	-	+	+
RU486	-	-	+	-	+

Figure 2

Southern analysis of mice tail DNA



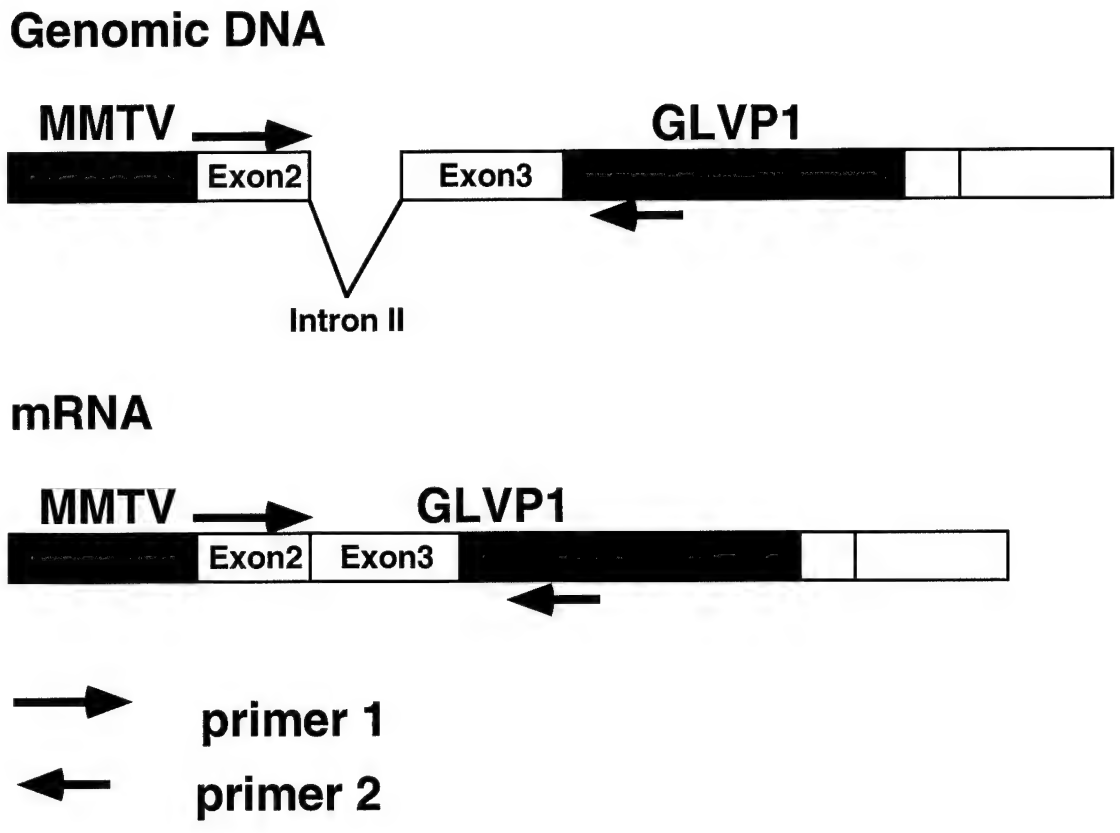
0, 1 & 10 : Copies of input transgenic vector added to non-transgenic mice tail DNA

+ : Positive control

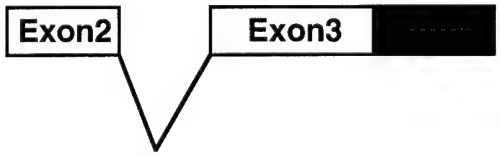
Arrow: Position of MMTV-KCR-GLVP1 transgene

Figure 3

Scheme of RT-PCR



Amplified PCR products

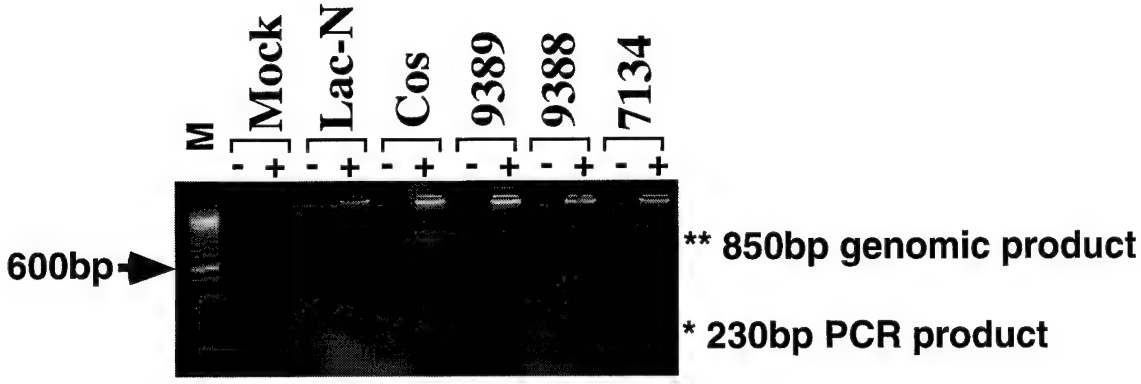


850 bp genomic product

230 bp RT-amplified product

Figure 4

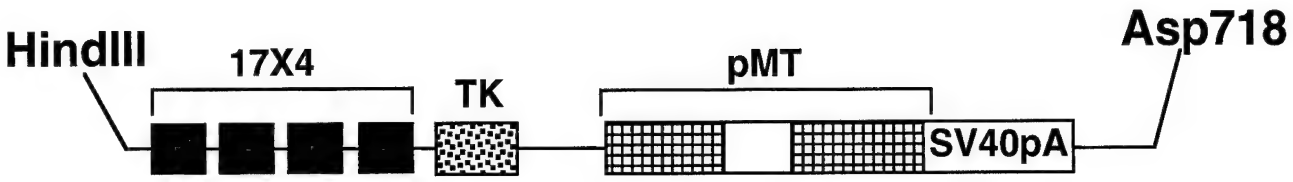
RT-PCR analysis of regulator expression



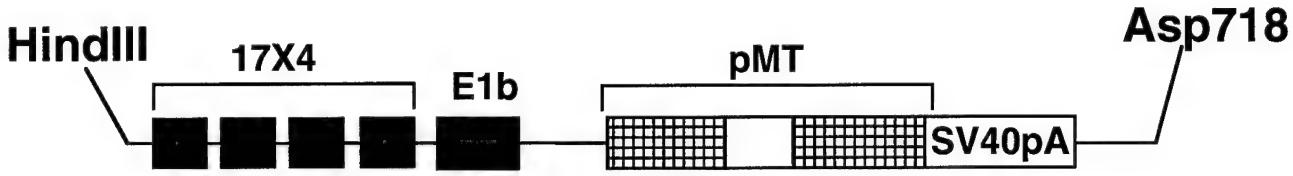
M: 100bp ladder
Mock: No input RNA
Lac-N: Non-transgenic lactating RNA
Cos: MMTV-KCR-GLVP1 transfected Cos cell RNA
7134, 9388 & 9399: MMTV-KCR-GLVP1 transgenic lines RNA
+/- : presence or absence of reverse transcriptase

Figure 5

Transgenic target vectors



17X4 TK-pMT-SV40pA



17X4 E1bTATA-pMT-SV40pA

Figure 6

MMTV-KCR-HAGLVP1bGHpA

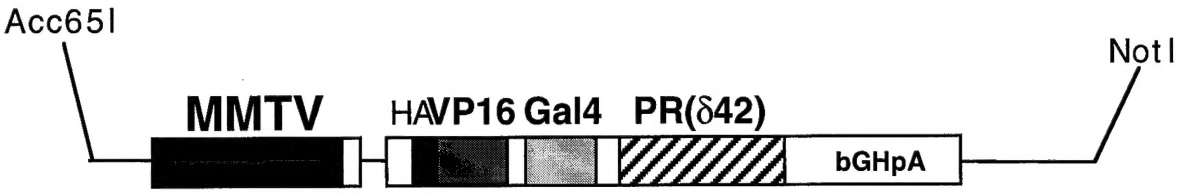


Figure 7

MMTV-KCR-HAGLVP1rBGpA

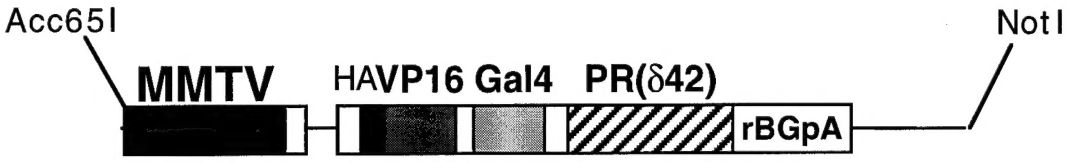


Figure 8

MMTV-KCR-GLVPC'rBGpA

Figure 9

Mice	Sex	Comments
7134	F	Low-expressor
9388	F	Non-expressor
9389	F	Non-expressor
9391	M	Breeding??
9801	M	Breeding
9804	M	Female is being tested

Table 1: Status of MMTV-KCR-GLVP1 mice